Remarks/Arguments:

Claims 18-28, presented hereby, are pending.

Claims 1-17 are canceled, without prejudice or disclaimer.

Present claims 18-20 contain the subject matter of claims 1 and 9, to, inter alia, limit the supporter cells, as well as the vectors used to transform the supporter cells, to specific embodiments. Present claim 21 limits claim 3 to the addition or removal of substances and/or heat as the external signal. While the former is properly exemplified in the specification by the use of a tetracycline inducible expression system, a person skilled in the art will readily understand that the latter feature refers to the various members of the family of heat shock promoters. These promoters are activated after a cell is exposed to heat-induced stress and are well known in the art, as shown by the enclosed on-line textbook excerpt of *Biochemistry*, 5th edition, by L. Stryer et al.

Claims 1-11, 13, 14, and 16 were rejected under 35 U.S.C. 112, first paragraph, as allegedly lacking enablement. Reconsideration is requested in view of the new claims presented hereby.

Present generic claim 18 provides for the use of specifically named supporter cells, in the method of growing stem cells as claimed; which, in the cases of keratinocytic stem cells (Examples 1-5) and tracheal epithelial cells (Examples 6-8) are described in detail in the instant specification. Based on these examples, a person skilled in the art is readily enabled to work the presently claimed invention with the other cell types named in claim 18.

The rejection further questioned whether embryonic stem cells can be cultured using the method according to the rejected claims and whether the differentiation into various specialized cell

types can be effected at will. In this context the parameter of cell morphology, alone, is allegedly deemed insufficient to prove maturation of the stem cells into terminally differentiated cells.

To address the foregoing allegation, submitted herewith are publications of Muth et al. ("Cell-based delivery of cytokines allows for the differentiation of a doxycycline inducible oligodendrocyte precursor cell line *in vitro*", J. Gene Med., 3, 2001, 585-598) and Elmshäuser et al. ("Characterization of a mouse tet-on glia precursor cell line *in vitro* and *in vivo* using the electrophysiological measurement", Journal of Physiology-Paris, 96, 2002, 329-338). Both references describe the culturing of a glial precursor cell line *in vitro* and its differentiation into oligodendrocytes and astrocytes after the addition of feeder cells. The undifferentiated stem cells are extensively characterized using, inter alia, antibody staining and electrophysiological measurements via the patch-clamp technique, confirming that the cells display the characteristics of stem cells.

Attention is specifically directed to Table 4 of the publication of Muth et al. and Figure 3 of the publication of Elmshäuser et al. Muth Table 4 and Elmshäuser Figure 3 show that the addition of human interleukin-3 secreting feeder cells and human interleukin-6 secreting feeder cells resulted in differentiation of the stem cells; whereas, the addition of non-cytokine secreting feeder cells led to the proliferation of the stem cells without stem-cell differentiation.

The methods of culturing stem cells described in both publications are used in embodiments of the presently claimed invention, thus proving that stem cells can be cultured and induced to differentiate, at will, using the method as disclosed and claimed in the present application.

Claims 1, 9, 11, 14, and 16 were rejected under 35 USC 112, 2^{nd} ¶, for allegedly being indefinite. Reconsideration is requested.

The present claims resolve the issues raised concerning "expression system" and "molecular bred" and "such as."

The allegation of unclarity of the term "spore-like" stem cell is incorrect, as the term is defined in the passage of the instant specification starting on page 3, last paragraph to page 4, fourth paragraph. The Examiner's definition of a claim limitation cannot conflict with the definition given in the specification. *In re Zletz,* 13 USPQ2d 1320, 1322 (Fed. Cir. 1989). The examiner must use the specification definition in construing the claims for comparison with the prior art.

> When the applicant states the meaning that the claim terms are intended to have, the claims are examined with that meaning, in order to achieve a complete exploration of the applicant's invention and its relation to the prior art.

Zletz, 13 USPQ2d at 1322.

Claims 1-11, 13, 14, and 16 were rejected under 35 USC 102(b) as allegedly anticipated by EP 0 753 574 A1 (Emerson). Reconsideration is requested in view of the present, newly submitted claims.

Emerson fails to disclose or suggest the specific supporter cells to which each of the present claims is limited. Instead, Emerson uses transfected fibroblasts, together with the stem cells and a suitable culture medium (Emerson, column 2, line 33, and column 10, line 9). The absence from a prior art reference of a single claim limitation negates anticipation. *Kolster Speedsteel A B v. Crucible Inc.*, 230 USPQ 81 (Fed. Cir. 1986). A reference that discloses "substantially the same

invention" is not an anticipation. *Jamesbury Corp. v. Litton Industrial Products, Inc.*, 225 USPQ 253 (Fed. Cir. 1985). To anticipate the claim, each claim limitation must "*identically* appear" in the reference disclosure. *Gechter v. Davidson*, 43 USPQ2d 1030, 1032 (Fed. Cir. 1997) (*emphasis added*).

Furthermore, Emerson, does not disclose or suggest a regulatable expression system that is started or stopped by the application of an external signal, as recited in the present claims. There is no hint in the experimental section of Emerson, starting in column 9, line 15, that the secretion of GM-CSF and IL-3 by the transfected fibroblasts is in any way externally regulatable. According to Emerson's description of the experiment, the transfected fibroblasts are transferred into culture and start secreting GM-CSF and IL-3 right away, without the addition or removal or any other substances. In contrast, the transformed supporter cells according to the presently claimed invention will start/stop the secretion exclusively in the presence/absence of an external signal and, therefore, are rightfully labelled <u>regulatable</u>.

Moreover, the presently claimed method offers several major advantages over the state of the art. Normally, non-regulated supporter cells need to be stopped from synthesizing DNA and from subsequent cell division. This was, usually, achieved by exposing a medium containing the supporter cells to high energy radiation or toxic chemicals. Irradiating supporter cells requires sophisticated equipment and usually results in subsequent apoptosis and necrosis of the supporter cells, which then release toxic substances into the medium. The released toxins, in turn, may interfere with growth of the stem cells.

Exposing the supporter cells to toxic chemicals requires extensive washing to avoid carry-over of the toxic chemicals into the medium housing the stem cells. Consequently, this constitutes a laborious and expensive procedure.

Furthermore, both exposure to radiation and exposure to toxic chemicals require a huge amount of back-up supporter cells. These back-up cells must be inserted into the culture once or twice per week, creating a recurrent disturbance for the growing stem cells.

The presently claimed invention provides a solution to these disadvantages, i.e., by employing supporter cells, while requiring neither the exposure to radiation or toxic chemicals, nor the continuous replenishment with back-up supporter cells, discussed above. DNA synthesis and cell growth of the supporters is controlled by the presence or absence of an external signal, which is not harmful to the stem cells. This results in prolonged lifetime of the supporter cells, and, thus, regular exchange of the supporter cells is avoided.

Request for Acknowledgment of Foreign Priority Under 35 USC 119

A claim to foreign priority under 35 USC 119 has been made to EP 99116533.3, filed August 24, 1999 (inventorship declaration of record, filed June 13, 2002) and the certified copy of the priority document was filed June 13, 2002 (copy of receipt card date-stamped "JUN 13 2002" attached).

Accordingly, request is made that the Examiner mark the next Office Action to acknowledge, both, the claim to §119 priority and receipt of the certified copy.

Favorable action is requested.

Respectfully submitted,

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MISSING PARTS OF APPLICATION
APPLICATION BRANCH

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The following has been received in the U.S. Patent & Trademark Office on the date stamped hereon:

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28.2.5. Multiple Transcription Factors Interact with Eukaryotic Promoters

The basal transcription complex described in Section 28.2.4. initiates transcription at a relatively low frequency. Additional transcription factors that bind to other sites are required to achieve a high rate of mRNA synthesis and to selectively stimulate specific genes. Upstream stimulatory sites in eukaryotic genes are diverse in sequence and variable in position. Their variety suggests that they are recognized by many different specific proteins. Indeed, many transcription factors have been isolated, and their binding sites have been identified by footprinting experiments (Figure 28.22). For example, *Sp1*, an ~ 100-kd protein from mammalian cells, binds to promoters that contain GC boxes. The duplex DNA of SV40 virus (a cancer-producing virus that infects monkey cells) contains five GC boxes from 50 to 100 bp upstream or downstream of start sites. The *CCAAT-binding transcription factor* (CTF; also called NF1), a 60-kd protein from mammalian cells, binds to the CAAT box. *A heat-shock transcription factor* (HSTF) is expressed in *Drosophila* after an abrupt increase in temperature. This 93-kd DNA-binding protein binds to the consensus sequence

5'-CNNGAANNTCCNNG-3'

Several copies of this sequence, known as the *heat-shock response element*, are present starting at a site 15 bp upstream of the TATA box. HSTF differs from σ^{32}, a heat-shock protein of *E. coli* (Section 28.1.2), in binding directly to response elements in heat-shock promoters rather than first becoming associated with RNA polymerase.

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RRSEARCH ARTICLE

Cell-based delivery of cytokines allows for the differentiation of a doxycycline inducible oligodendrocyte precursor cell line *in vitro*

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Abstract

Background Stem cells, having the property of self renewal, offer the promise of lifelong repair of damaged tissue. However, somatic tissue-committed primary stem cells are rare and difficult to expand *in vitro*. Genetically modified stem-like cells with the ability to expand conditionally provide a valuable tool with which to study stem cell biology, especially the cellular events of proliferation and differentiation. In addition, stem cells may be appropriate candidates for therapeutic applications.

Methods Double transgenic mice possesing SV40 T antigen (Tag) under the control of the reverse tetracycline-transactivator (rtTA) were used to establish cell lines. One brain cell line was partially characterized by DNA sequencing, morphology, antigen expression using flow cytometry, confocal microscopy, and electrophysiology using the patch clamp technique. Cell cycle analysis was performed using propidium iodide staining; cell viability and H^3-thymidine incorporation assays. The ability of this cell line to differentiate was assessed by confocal microscopy following co-culture with stem cells secreting cytokines.

Results We report here the establishment and partial characterization of a cell line derived from the brain tissue of rtTA-SV40 Tag transgenic mice. Analysis of the morphology and antigen markers has shown that this cell line mimics some aspects of primary glial precursors. The results of electrophysiology are consistent with this and suggest that the cell line is derived from O2A glial precursor cells. Cell cycle progression of this cell line is doxycycline-dependent. In the absence of doxycycline, cells become apoptotic. Differentiation into mature type 2 astrocytes and (precursor) oligodendrocytes can be induced upon withdrawal of doxycycline and addition of epithelial stem cells secreting cytokine, such as hIL3 (human Interleukine 3) or hIL6 to the culture. In contrast, co-culturing with hCNTF (human Ciliary NeuroTrophic Factor)-secreting epithelial stem cells did not induce them to mature into progeny cell types.

Conclusion The differentiation of this O2A glial precursor line does not occur automatically in culture. Additional external help is required from the cell-based delivery of appropriate transgenic cytokines. Withdrawal of doxycycline from the culture medium removes the proliferation signals and induces a fatal outcome. Copyright © 2001 John Wiley & Sons, Ltd.

Keywords proliferation versus differentiation; doxycycline inducible stem/precursor cells

Introduction

Stem cells exist for the lifetime of an organism and can renew themselves for maintaining the stem cell pool and can differentiate to progenies with limited lifetimes for organ-specific functions. Stem cells may be totipotent like mouse embryonic stem cells, pluripotent like hematopoietic stem cells and neuronal-glial stem cells, or unipotent like keratinocytic stem cells. Somatic stem cells are rare and often quiescent. They can become cycling precursor cells. Stem cells may also be plastic and may migrate freely [1]. Until now it has been quite difficult to grow these rare somatic stem cells. Recently there have been major breakthroughs. Conditional expansion of some lineages of stem cells has become possible and this provides a very promising tool for stem cell based tissue therapy.

Neuronal and glial stem/precursor cells

Neurodegenerative diseases of the central nervous system (CNS), such as Parkinson's disease, are due to a defect in dopaminergic neurons. Motor function in Parkinson's patients was recently restored by fetal human brain cell transplantation. Multiple sclerosis is a demyelination disease of unknown cause. Autoimmune destruction of myelin kills the oligodendrocytes that form myelin and also neurons. In animal models of demyelination, transplantation of oligodendrocyte stem/precursor cells produces many functional oligodendrocytes. Thus glial stem/precursor cell transplantation is thought to be the most promising way to treat neurodegenerative diseases. As only cell replacement will restore lost function, cell transplantation is crucial for treating such diseases. The discovery of CNS stem cells in rodents and human fetuses opens new prospects for neural cell transplantation, although low availability and ethical problems weigh against using human fetal CNS stem/precursor cells [2–4]. Precursor cells of rodent or fetal human origin that are destined for neuronal and glial lineages can be cultured in suspension. More specifically, spheres of brain stem cells can form, and expand, in a serum-free medium containing factors such as EGF, bFGF-2 and LIF. Glial stem cells seem to require bFGF, PDGF and GDNF for growth and differentiation. There are controversial reports on the effects of CNTF and NGF on glial cells. Several markers are available to characterize these cells at different stages of differentiation [5]. Rodent and human fetal stem/precursor cells are quite easy to expand in culture, while human adult stem/precursor cells are extremely difficult to grow, since they do not respond to soluble factors so far examined [6]. There are several rodent cell lines available [7–9], possessing certain biological properties mimicking primary glial and neural stem/precursor cells.

Tet-on system

A tightly regulated inducible system is required for the study of spatial and temporal gene expression. A limited amount of gene expression under defined conditions is desirable in many applications. Although numerous inducible systems are currently available *ex vivo*, very few behave as expected. Common problems encountered include leakiness, toxicity and positioned effects. Recently, the tetracycline regulatable/dependent system has become popular, because it offers the promise of regulatable gene expression *in vitro* and *in vivo*. It exists as two systems, the tet-off [10] and tet-on [11] systems. In the tetracycline inducible vector, the gene of interest is placed under the control of a tetracycline responsive element (tet-operator) upstream of a minimal promoter (tk). The gene is transcribed when the activator rtTA (the reverse tet-repressor fused to the VP16 activation domain), which is driven by a ubiquitous promoter-tk, binds to the tet-operator in the presence of tetracycline and its analog, doxycycline.

Controversial reports of current doxycycline inducible systems and the subsequent modification to improve the existing tetracycline inducible system have been documented by us [1] as well as by others. We report here partial characterization of one cell line derived from the brain tissue of the crossed offspring of tet-inducible transgenic mice using the original vector. The rtTA mice were made from the doxycycline inducible tk-rtTA plasmid vector [11,12] and the responding mice containing reporter transgenes were made using the teto-SV40 T antigen plasmid vector [13]. Despite some negative and controversial data using this inducible system, this cell line behaves as expected. Transgenes are expressed under permissive conditions in the presence of doxycycline, and are not expressed without doxycycline.

Materials and methods

Culture condition for cell growth

Cells were grown in complete RPMI-1640 (Gibco) supplemented with 10% heat-inactivated, preselected fetal calf serum (Boehringer, Mannheim), and freshly prepared doxycycline at 1 µg/ml. Cells were passaged weekly, following trypsinization (Gibco), to detach cells from plastic culture petri dishes and transfer to new dishes. The cultures were kept at 37°C in a humidified incubator with air and 5% CO_2.

PCR and DNA sequence analysis

1×10^4 cells were harvested from culture, washed once with PBS, resuspended in 100 µl H_2O, boiled for 15 min and then frozen at −20°C.

Primers of rtTA-5′ (5′-GAATTCATATGTCTAGATTAG AT-3′) and rtTA-3′ (5′-CACCTTGGTGATCAAATAATTC-3′), were used to analyse the rtTA motif yielding a fragment of

551 bp corresponding to the rtTA sequence position 766–1317 in plasmid pUHD17-1 ([11]; sequence accession: (<<http://www.zmbh.uni-heidelberg.de/Bujard/rtTA/pUHG17-1.htm TARGET="_blank">http://www.zmbh.uni-heidelberg.de/Bujard/rtTA/pUHG17-1.html>).

PCR conditions were the following: to 1 µl of boiled cells were added 1 µl of 10 × reaction buffer (Promega, Madison), 2 µl each of the two primers, 2 µl of dNTP mix (2.5 mM), 2 µl of 2.5 mM $MgCl_2$, 0.2 µl of Taq polymerase (Promega, Madison, 5 units/µl) and 17.8 µl H_2O. PCR conditions were: 94°C for 0.5 min, 57°C for 1 min and 72°C for 1 min, for a total of 35 cycles. After the PCR reaction, DNA amplified from the transgenic cells was subjected to analysis, using 2% agarose gel eletrophoresis. The DNA sequence was derived from the PCR fragment directly from both ends by using the primers rtTA-5′ and rtTA-3′, by custom sequencing.

Cell morphology upon limiting dilution experiments

Limiting dilution experiments were performed to select single cells in individual wells of 96-well flat bottomed, tissue culture plates (Renner, Dannstadt-Schauernheim) and to follow the fate of single cells based on morphology. Ten-fold dilutions of cells in culture medium, starting from a concentration of 1×10^6/ml, were seeded in a row of 12 wells in 96-well tissue culture plates. Serial dilutions were made until single or no cells had been achieved in the wells [14,15]. Cell morphology was examined and recorded every three days for 21 days.

Immunostaining and FACS analysis

Cells were stained with (monoclonal) antibody (mAb) of glial and neuronal lineages, and FITC-labeled second antibody. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin at room temperature (RT) for 30 min each for intracellular staining. Cells were washed with PBS twice in between. Then, 10% FCS in PBS was used to block non-specific binding to cells at RT for 30 min. Unless specifically indicated, antibodies used in this study were diluted 1:100. In all solutions for making dilution of antibodies, 10% FCS in PBS was included to block non-specific binding to cells. mAb's used for FACS were the following: 513; mIgG (mouse IgG) anti-513 (gift of J. Trotter, 1:20 dilution) and FITC. G (Goat) anti-mIgG (Jackson, West Grove); A2B5: mIgM anti-A2B5 (Boehringer, Mannheim, 5 µg/ml) and FITC-G anti-mIgM F(ab)'2 (Silenus, Hawthron); O4: mIgM anti-O4 (Boehringer, Mannheim, 10 µg/ml) and FITC-G anti-mIgM F(ab)'2; O10: mIgM anti-O10 (gift of J. Trotter, 1:5 dilution) and FITC-G anti-mIgM F(ab)'2; O1: mIgM anti-O1 (gift of J. Trotter, 1:50 dilution) and FITC-G anti-mIgM F(ab)'2; Oct 6: R (Rabbit) IgG anti-Oct 6 (rabbit anti-recombinant Oct-6 protein and preabsorbed using Octamer proteins, gift of H. Schoeler, 1:1000 dilution), and FITC m anti-R IgG (SBA, Birmingham); GFAP: mIgG anti-GFAP (glial fibrillary acidic proteins, Boehringer, Mannheim) and FITC G anti-mIgG (SBA, Birmingham); NF68KDa: mIgG anti-NF68Kda (neurofilament 68 KDa, Boehringer, Mannheim) and FITC G anti-mIgG. Stained cells were measured and analyzed using a Becton Dickinson FACS scanner.

Immunostaining and confocal microscopy

For measuring the expression of antigens in the cells, we performed antibody-specific immunofluorescent labeling and evaluated using confocal microscopy. Cells were seeded onto coverslips and cultured for two days before the evaluation. Cells were fixed with 4% paraformaldehyde in PBS at RT for 30 min, washed twice with PBS (in between steps), nonspecific binding sites were blocked with 2% FCS in PBS at RT for one hour, then stained with the following antibodies: anti-SV40 T antigen: m IgG2a anti-SV40 T antigen (Santa Cruz Biotech, California), biotin-G anti-mIgG2a (SBA, Birmingham), and FITC-streptavidin (Amersham, Amersham). Afterwards, the coverslips were embedded in Moviol containing 100 µg/ml DABCO (Vectashield, Bector, Burlingame).

Neuronal lineage was identified with NF68KD: mIgG anti-NF68KDa and FITC-G anti-mIgG; OMP (olfactory membrane protein, [16]): G anti-OMP and Texas Red (TR) R anti-G IgG (Jackson, West Grove); nestin: mIgG anti-nestin (gift of E. Cattaneo, 1:1 dilution) and FITC G anti-mIgG for precursors. Glial lineage was identified with GFAP: mIgG anti-GFAP and FITC-G anti-mIgG; O4: mIgM anti-O4 and FITC-G anti-mIgM F(ab')2; 513: mIgG anti-513 and FITC-G anti-mIgG; A2B5: mIgM anti-A2B5, biotin-G anti-mIgM F(ab)'2 and TR-streptavidin; GalC: mIgG anti-GalC (Galactocerebrosid, gift of J. Trotter, 1:10 dilution) and FITC-G anti-mIgG; O6: RIgG anti-recombinant Oct-6 protein and FITC-m anti-R, or TR-G anti-R IgG.

Immunofluorescence was evaluated using confocal scanning laser microscopy. A Leica DM IRBE microscope equipped with a 63 × Plan Apochromate objective was used in this study. Analysis of the antigens, stained by using the TR- and FITC-labeled secondary antibodies was performed by using the TRITC- and FITC-fittings at a pinhole size of 0.45. Relative amplification of the FITC and TRITC emissions was 1:0.97. Laser excitation energy and amplification of FITC and TRITC emissions were left constant within each experiment to allow the comparision of the different samples.

Electrophysiology

For electrophysiological recordings coverslips with adhered cells were placed in a chamber mounted on the stage of a Zeiss microscope (Axioplan, Zeiss, Oberkochen, Germany) and fixed in the chamber using a U-shaped platinum wire. The chamber was continuously perfused with HEPES buffered bath solution and

substances were added by changing the perfusate. Membrane currents were measured with the patch-clamp technique in the whole-cell recording configuration [17]. Current signals were amplified (EPC-9 amplifier, HEKA, Lambrecht, Germany), filtered at 3 kHz and sampled at 5 kHz by an interface (HEKA) connected to a PC system, which also served as a stimulus generator. All patch clamp data analysis was performed using the WinTida software package (HEKA). The resistance of the patch pipettes was 5–6 M. The bathing solution contained (in mM) NaCl, 150; KCl, 5.4; $CaCl_2$, 2; $MgCl_2$ 1; HEPES, 5; and glucose, 10. The pH was adjusted with NaOH to 7.4. The pipette solution contained (in mM) KCl, 130; $CaCl_2$, 0.5; EGTA, 5; $MgCl_2$, 2; HEPES, 10, Ca_2+ activity was calculated to be approximately 11 nm. The pH was adjusted with NaOH to 7.2 [18,19].

Cell cycle analysis

Cell cycle analysis was performed on cells harvested after stimulation with doxycycline for 2–8 days. Cells were fixed with 4% paraformaldehyde, permeabilized using 0.1% saponin, and incubated with a propidium iodide (50 µg/ml) and RNase (25 µg/ml) solution for 30 min at RT. Flow cytometry analysis was performed on FACScan (Becton Dickinson). A total of 10^5 cells in each sample were acquired. (Cell doublets were excluded on FL2-width/FL2-area dot plots). The studied cells were selected by gating FL1 positive cells. Events were plotted on a one-parameter histogram on a linear scale. The percentage of cells in each region (G_0/G_1, S, G_2/M) was determined with the MODFIT LT 2.0 program (Becton Dickinson). Results were expressed as the percentage of cells in a cell cycle compartment [19a].

H^3-thymidine incorporation assay

1×10^4 cells were cultured in triplicates for four days in 96 well microtiter plates (Costar, Cambridge, MA), in 100 µl of RPMI 1640 complete medium with 10% FCS. The [H^3] deoxythymidine (dThd) was added from day 3 to day 4. [H^3] dThd incorporation was measured following a 16 h pulse. The data were expressed as mean of triplicate cultures, SD < 10% (not shown).

Cell viability assay

Cell counts were performed following the protocol described in [20] using a Hemacytometer. The viability was determined by trypan blue exclusion. Percentage viable cells = number of viable cells/number of viable cells + number of dead cells × 100%.

Co-culture glial precursor cells and growth factor secreting feeder cells

Cells were seeded onto glass coverslips to achieve 70% confluence after two days. Coverslips of two sets of cells were placed in 10 ml petri dishes so that there was no direct contact between coverslips. Cells were co-cultured in 10 ml of RPMI complete medium with 10% FCS (Boehringer, Mannheim, Germany) for nine days. Cells were then harvested, washed with PBS, fixed with 4% paraformaldehyde in PBS and stained with FITC or TR labeled mAbs against epitopes expressed in the gliogenesis lineage, as described in the section of confocal microscopy. The feeder cells were doxycycline regulatable mouse epithelial stem cell lines and included the untransfected and the hCNTF-, hIL3-, or hIL6-stably transfected and secreting cell clones [Elmshaeuser *et al.*, submitted]. *S1 and S2 facilities are available in the Institute [Aktenzeichen (S2): 32-GT/530 06.05.02G, UniGI1/93, (S1): 32-53e621-GenA-UniGI 1/90].*

Results

Isolation of a cell line from brain of rtTA x SV40 T antigen double transgenic mice

Cells from 10 tissues of double transgenic mice carrying the ptk-rtTA x teto-PhCMV-SV40 Tag constructs were isolated. In the presence of doxycycline, SV40 Tag is expressed and cells should be rendered immortal. Conversely, in the absence of doxycycline, SV40 Tag is switched off and cells are apoptotic or prone to differentiation ('tet-on system'). Cells obtained from the embryonic tissues of these mice could be cultured for several months, but then perished. Three initial attempts to culture cells from such adult mice were unsuccessful. We then obtained 10 tissues from an adult double positive mouse at the fourth attempt. Immunohistology staining of tissue cryosections revealed that 9 of the 10 tissues expresses SV40 Tag. However, only cells from five kinds of tissue that survived the weekly passage were still alive after three months. We report here partial characterization of one cell line established from the brain tissue of one such tet-on transgenic mouse. The rest of the tissues and cells were frozen, awaiting future work. Although we cannot yet estimate the frequency of establishable cell lines from these double transgenic mice, Efrat has reported the development of β-cell tumors to be 1–2% of the islets of rat insulin promoter (RIP) rTA x teto-PhCMV-SV40 Tag double transgenic mice [13].

DNA sequencing of PCR products

A PCR fragment containing the rtTA motif derived from the DNA extracted from this cell line was sequenced. The sequences were compared with the published DNA sequences [11] and considered to be correct, as for the published data of Bujard's group (data not shown).

Fate of single cells

Under the microscope, two main morphological types of cells could be observed when cells were grown in conventional plastic petri dishes: (1) long cells with an elongated thin cell body, called long thin cells in this study; and (2) very flat cells with a round cell body, called round flat cells in this study. Single cells of either long thin or round flat cell type in individual culture wells were selected by limiting dilution experiments and their fate was followed.

Mixed cell types were constantly present whenever a few cells were seeded in the wells, and they proliferated in a way similar to parentals. Single cells were present in some wells and their fate is represented in Figure 1: a long thin cell could round up to a flat cell over the observation time of 21 days, and a round flat cell could become more radial (differentiated). The progeny of a long thin cell were either two long thin cells (symmetric division), or one long thin cell and one round flat cell (asymmetric division), or two round flat cells (symmetric division). The progeny of a round cell were two round flat cells (symmetric division), or two long thin cells (symmetric retro-differentiation). Since cell division occurs in a pattern of symmetric, asymmetric, and symmetric retro-divisions, it may provide a cellular basis for studying the molecular mechanism underlying such events.

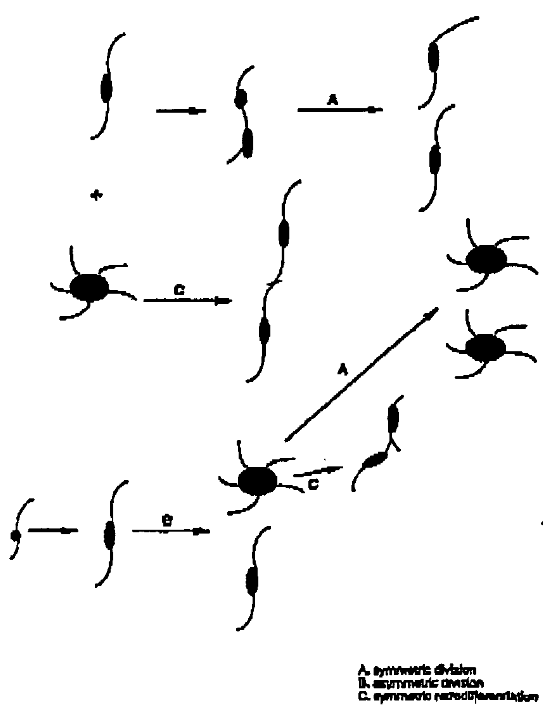


Figure 1. A cartoon of cell division pattern was drawn based on the morphology of the two cell types observed under the microscope, following the fate of single cells in 96-well dishes, cultured for two weeks

All single cells and their progeny survived 20 days, then, died after three weeks, while few or multiple cells continued to grow and could be passaged and expanded. The results suggest a cross-feeding effect between the cells and implies that autocrine cytokines play a role in supporting the growth of such cells, in addition to the doxycycline-dependent cell proliferation mechanism.

Characterization of a doxycycline inducible cell line using flow cytometry and confocal microscopic analysis

The phenotype of the cell line was determined by staining with antibodies directed against known membrane and intracellular markers of glial and neuronal lineages and flow cytometry analysis. The data in Figure 2 show that this cell line is A2B5$^+$, O10$^+$, O1$^+$, 513$^-$, 04$^-$, Oct 6$^-$, GFAP and NF68KDa$^-$. Among the positively stained cells, 33% were A2B5$^+$, 19.4% were O10$^+$, and 77% were O1$^+$.

To further characterize for surface markers, cells were seeded onto coverslips and grown in medium with doxycycline for two days, immunostained and analyzed by confocal microscopy. Figure 3 shows that most cells were O4$^+$ (panel 3), O4$^+$ (green) Oct 6$^+$ (red) (panel 7), SV40 Tag$^+$ (panel 5), OMP$^+$ (panel 9), nestin$^-$ (panel 8), a few spindle cells were also positive for GFAP (panel 2) and NF68kDa in cytoplasm.

It is interesting that O4 was negative using cells freshly isolated from plastic petri dishes and analyzed by flow cytometry, and they were positive when cells were seeded onto glass coverslips for confocal microscopy (Figure 3–3) and for electrophysiology (next section). It implies that the glass surface triggered the differentiation of such cells from O4$^-$ to become O4$^+$. Taken together, flow cytometry and confocal microscopy analyses show that these cells possess characteristics of glial precursor cells (A2B5$^+$, 04$^{-/+}$, O1$^+$, O10$^+$).

Electrophysiological study of a doxycycline inducible cell line

In order to further characterize this cell line, we performed electrophysiological measurements using the patch-clamp technique. Cells were grown on coverslips for two days with or without doxycycline. For 22 of the 24 cells investigated, a whole cell configuration could be achieved. These cells displayed a membrane potential and current patterns common to cultured glia cells (Figure 4). Some round flat cells showed an oligodendroglia-like morphology. The results of electrophysiology and morphology support the data for surface marker and biology, therefore these cells are likely to be of glia precursor cell lineage. It is most likely an O2A oligodendrocyte-precursor cell line. Figure 5 shows a possible location of this cell line in glio-genesis.

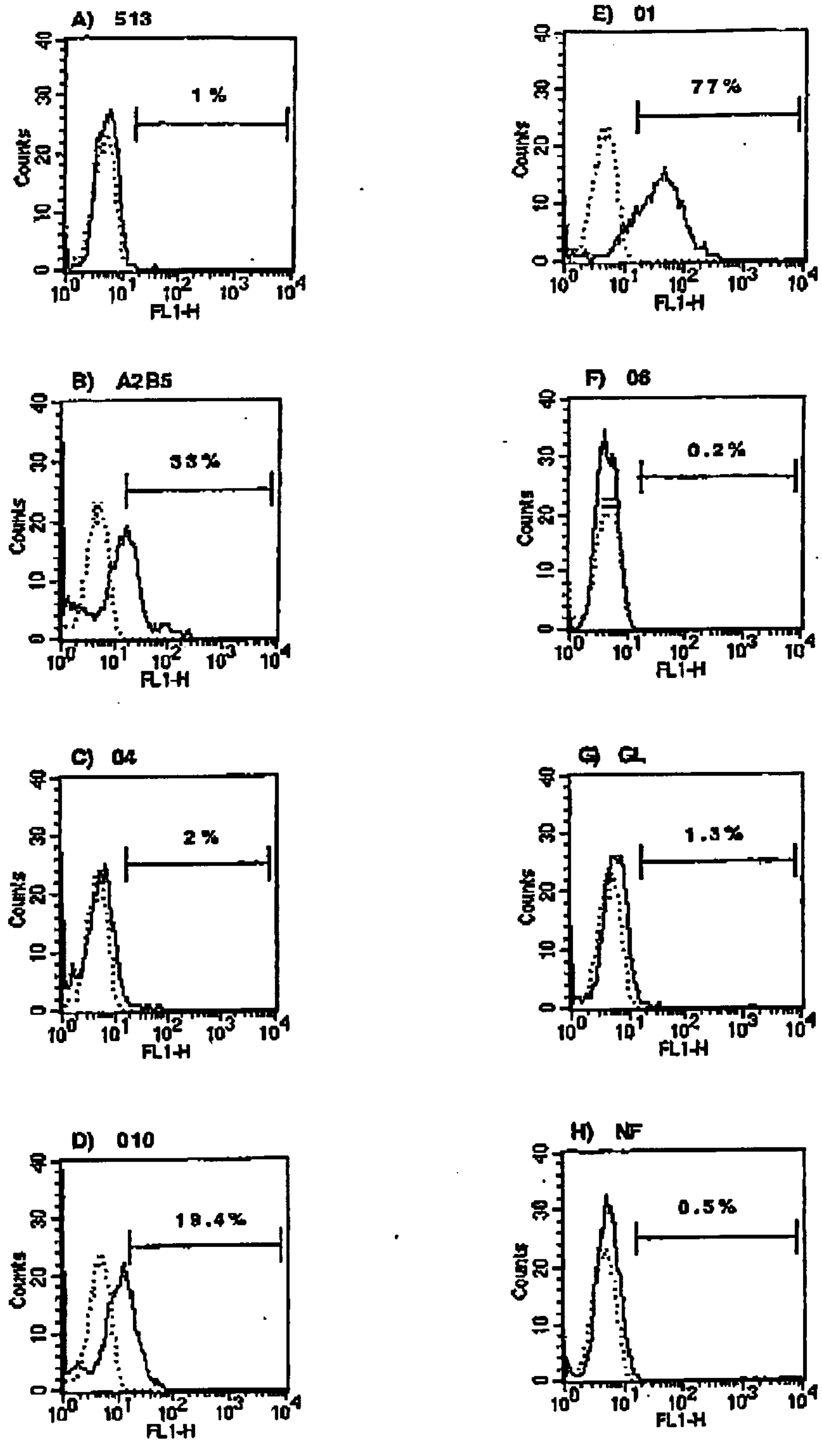


Figure 2. FACS analysis of a cell line. Cells were fixed with 4% paraformaldehyde and permeabilized with saponin for intracellular staining. Cells were stained with FITC-labeled mAb's of glial and neuronal lineages. Panel A: anti-S13, panel B: anti-A2B5, panel C: anti-O4, panel D: anti-O10, panel E: anti-O1, panel F: anti-Oct 6, panel G: anti-GFAP, and panel H: anti-NF68KDa.

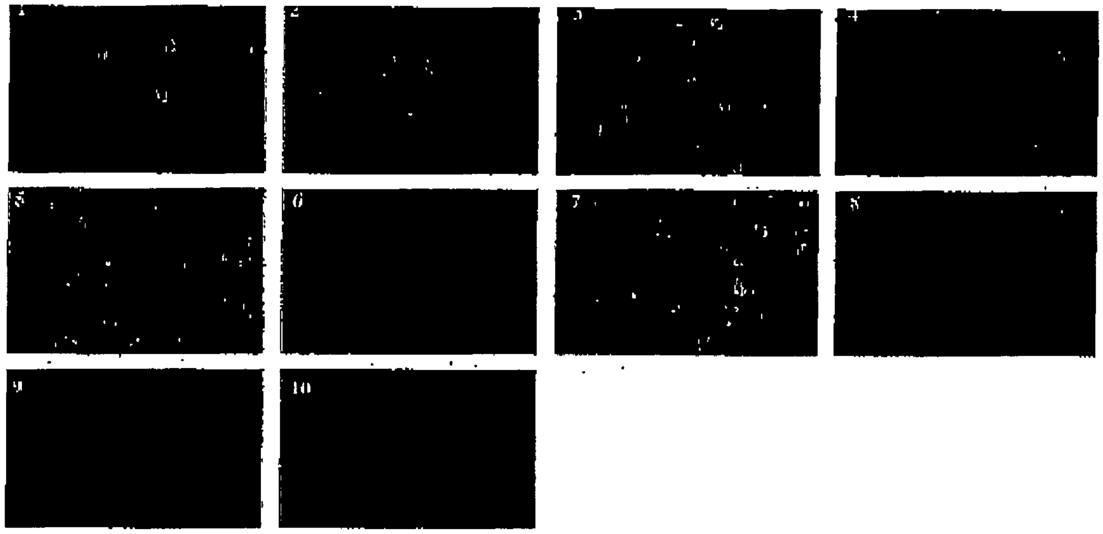


Figure 3. Confocal microscopic photographs of a cell line. Cells were seeded onto coverslips and cultured for two days before the evaluation. Cells were rinsed in PBS and fixed with 4% paraformaldehyde in PBS, and stained with the following antibodies: Panel 1: NF68KDa; mIgG anti-NF68 kDa and FITC-G anti-mIgG. Panel 2: GFAP; m-anti-GFAP and FITC-G anti-mIgG. Panel 3: O4; mIgM anti-O4 and FITC-G anti-mIgM F(ab')2. Panel 4: Oct 6; R-IgG anti-Oct 6 and FITC G-anti-R IgG. Panel 5: anti-SV40 Tag; mIgG2a anti-SV40Tag, biotin-G anti-mIgG2a, and FITC-streptavidin. Panel 6: second antibody control. Panel 7: O4 and O6; anti-O4 and FITC-G anti-mIgM-FITC (green), R-anti-Oct-6 and TR-G anti-R IgG (red). Note the double labeling (yellow = green plus red). Panel 8: nestin; mIgG anti-nestin and FITC-G anti-mIgG. Panel 9: OMP; G-anti-OMP and TR-m anti-G IgG. Panel 10: negative control

Cell cycle progression analysis using the propidium iodide staining method

Cells were grown and passaged weekly with 1 μg/ml doxycycline included in the medium. For cell cycle analysis, cells were harvested, washed twice with medium without doxycycline and cultured for 24 hours in the absence of doxycycline. Culture medium was then replaced with fresh medium, either medium alone or medium with 1, 3 or 10 μg/ml of doxycycline, and cultured for 2–8 days. Cell cycle analysis was performed as indicated in the Materials and Methods section. As shown in Table 1, without doxycycline in the medium, the percentage of cultured cells in the S compartment is

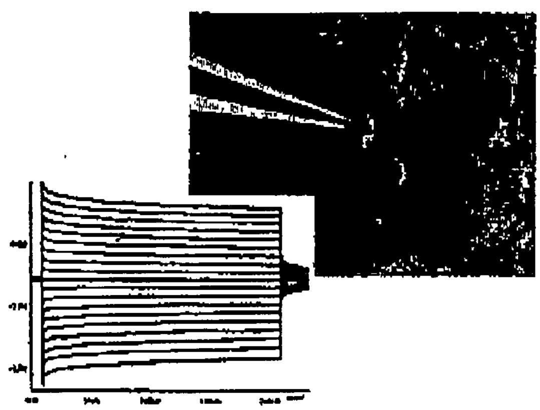


Figure 4. Electrophysiological and morphological properties of a round flat cell. The cell displays an oligodendroglia-like morphology and is characterized by a symmetrical pattern of non-inactivating outward and inward currents elicited by de- and hyperpolarizing voltage steps (from −160 to +20 mV). The membrane was clamped at −70 mV. Bars denote 1 nA (vertical) and 50 ms (horizontal)

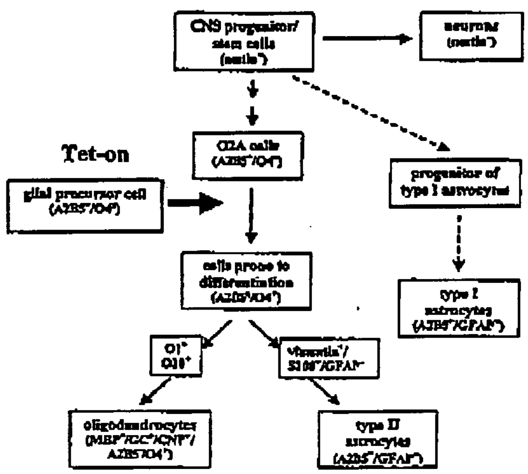


Figure 5. Gliogenesis: a doxycycline-inducible cell line, possibly of O2A cells or the immediate progeny. The lineage development of neuronal and glial cells are drawn as a cartoon, based on published data [5,22]

Table 1. The effect of doxycycline on the distribution of glial progenitor cells in the cell cycle compartment in culture for 2 to 8 days. Analysis of cell cycle progressing using the propidium iodide staining method

	Percentage of cells in cell cycle compartment											
Doxycycline (μg/ml)	0			1			3			10		
Day	G_0/G	S	G_2/M	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
2	52.0	45.3	2.7	69.4	30.6	0.0	34.9	65.1	0.0	53.9	15.7	30.4
3	53.6	38.7	7.7	65.0	25.4	9.6	14.4	85.6	0.0	100.0	0.0	0.0
4	67.8	10.9	21.3	50.5	49.5	0.0	68.7	30.7	0.6	nd	nd	nd
8	79.7	0.0	20.3	50.6	44.7	4.7	77.4	6.7	15.9	78.9	2.8	18.3

nd = not done.

45.3% on day 2, 38.7% on day 3, 10.9% on day 4, and 0% on day 8. Thus, after depleting doxycycline for 8 days, cells were no longer in the S phase. Instead, they were found to be in the G_0/G_1 compartment (79.7% on day 8 vs. 52.0% on day 2), and very significantly, in the G_2/M compartment (20.3% on day 8 vs. 2.7% on day 2).

The concentration of doxycycline in the culture medium plays a critical role in cell cycle distribution. After 8 days culturing with 0, 1, 3, 10 μg/ml of doxycycline, the percentage of cells in the S compartment is 0.0%, 44.7%, 6.7% and 2.8%, respectively. Inclusion of 1 μg/ml of doxycycline in the culture medium drives cells into the S phase (44.7% compared with 0% in the absence of doxycycline). The distribution of cells in each compartment is rather constant of all time points measured, ranging from 25.4% to 49.5%. In fact, 1 μg/ml doxycycline is the concentration used to propagate cells weekly for establishing and for maintaining the cell line in culture.

Doxycycline at 3 μg/ml in culture medium exhibits a biphasic effect: a growth promoting effect in a short term (2–3 days) culture, and a growth inhibitory effect in a prolonged (8 days) culture. The concentration of 10 μg/ml of doxycycline is inhibitory at all time points measured.

The above data indicate that this cell line depends on the presence of doxycycline in the culture medium for growth promoting effect. After 8 days starvation, no cells are in the S phase, they either remain in the G_0/G_1 phase, or are retained in the G_2/M compartment. In the presence of 1 μg/ml doxycycline, a great proportion of cells leave the G_0/G_1 and G_2/M compartments to enter the S phase, the distribution of cells in each compartment is similar to many other cell types measured. Increasing the concentration further, doxycycline becomes toxic to these cells.

Cell proliferation using H^3-thymidine incorporation assay

The effect of different concentrations of doxycycline in cell growth was further analyzed using H^3-thymidine incorporation and cell viability assays. Cells were starved of doxycycline for 1 week, harvested and seeded in 96-well culture plates overnight. The medium was replaced with either fresh medium alone, or fresh medium with doxycycline. The concentration of doxycycline ranged from 0.1–30 μg/ml. Cells were cultured for 4 days and pulsed with H^3-thymidine from day 3 to day 4.

As shown in Table 2, when 0–1 μg/ml of doxycycline was included in culture medium, there was a steady increase of H^3-thymidine incorporated into the DNA of the tested cells. It reached a 34-fold increase, comparing 639 cpm per culture with 0 μg/ml of doxycycline and 21 855 cpm per culture with 1 μg/ml of doxycycline. Increasing the concentration of doxycycline further in culture medium becomes inhibitory to cells, most drastically with 10 and 30 μg/ml of doxycycline in culture medium.

Cell viability using a trypan blue exclusion assay

Triplicate cells in 96-well plastic tissue culture plates of concentration 1×10^5/ml, 100 μl each, were cultured overnight. The medium was replaced with fresh medium, or fresh medium with doxycycline of 1, 3 or 10 μg/ml. Cells were harvested, and cell count was determined in the presence of trypan blue in a hematocrit chamber on days 1, 2, 3 and 8. As shown in Table 3, when the cell viability was measured on days 1, 2 and 3, there was no apparent difference between cells treated with 1 and 3 μg/ml doxycycline (panels B and C) versus cells without doxycycline (panel A). The percentage of viable cells treated with 10 μg/ml of doxycycline on day 1 was lower than that of other concentrations, but the significance is not clear since the data of other time points of this concentration were comparable to others.

The results of propidium iodide cell cycle analysis, H^3-thymidine incorporation, and cell viability assays of these cells show that doxycycline at the appropriate

Table 2. H^3-thymidine incorporation assay. H^3-thymidine incorporation assay of a cell line cultured without and with doxycycline for four days. H^3-thymidine was included in the culture from day 3 to day 4 for 16 hours. The concentration of doxycycline was from 0–30 μg/ml in culture. The amount of H^3-thymidine incorporated into cells was measured and the data are expressed as the average amount of H^3-thymidine incorporated into cells (cpm) of triplicate culture (mean ± SD)

Doxycycline (μg/ml)	Mean ± SD (cpm)	
0.0	2707	1514
0.3	8396	4466
1.0	13 348	6375
3.0	8014	4926
10.0	3682	2199
30.0	622	1334

Table 3. Viability test. Cell viability of a cell line, based on a trypan blue exclusion assay. Triplicate culture wells were each seeded with 1×10^4 cells in each well. Doxycycline of different concentrations (0, 1, 3, 10 μg/ml) was included in the RPMI-1640 culture medium. Cells were harvested on days 1, 2, 3, 4 and 8 after seeding, and (viable) cell numbers in each well were determined using a hematocrite chamber. The viability of cells was calculated from an avearage of triplicate wells, and the percentage was determined by the formula: % viable cells=viable number of cells in well/total number of cells in well ×100%

	Viability (%)			
Doxycycline (μg/ml)	Day 1	Day 2	Day 3	Day 8
0.0	44.4	60.0	58.3	63.9
1.0	44.4	53.8	57.1	66.7
3.0	37.5	50.0	54.4	68.5
10.0	30.0	57.1	55.5	72.2

concentration (1 μg/ml) induces these cells to enter the S phase from the G_0/G_1 compartment and to leave the G_2/M compartment. The growth-promoting effect of doxycycline on this cell line is demonstrated by the increased percentage of cells in the S phase (Table 1) and the increased amount of H^3-thymidine incorporated into DNA (Table 2), but doxycycline does not affect cell viability (Table 3). Higher concentrations of doxycycline inhibit entry of cells into the S phase and proliferation. Based on these biological criteria, this cell line is characterized to be a doxycycline inducible (tet-on) cell line.

Differentiation of a glial precursor cell line *in vitro* upon withdrawal of doxycycline

In order to address the question of proliferation versus differentiation, coverslips of glial precursor cells were cultured without doxycycline or with doxycycline, either alone, or with feeder cells also seeded onto coverslips. The coverslips were placed in the same petri dish at a distance so as to avoid direct contact between the two types of cells. The feeder cells were either an untransfected tet-off stem cell line, or tet-off stem cell lines secreting hCNTF, hIL3 or hIL6. After 9 days culture, the glass coverslips containing glial precursor cells were harvested, fixed and stained with fluorescent labeled mAb's for confocal microscopic image analysis.

In cultures with doxycycline (Table 4, lower panel), glial precursor cells did not yield either (precursor) oligodendrocytes, or type 2 astrocytes regardless of the combination tested so far, i.e., glial precursors alone, or co-cultured with untransfected tet-off feeder cells or with tet-off feeder cells transfected with hCNTF, hIL6, hIL3, (but secretion was turned off by doxycycline).

As shown in Figure 6 and Table 4, culturing glial precursor cells in medium alone (without doxycycline and without feeder cells) yielded 513^- $A2B5^-$, round flat and dying cells, and the long thin $A2B5^+$ cells could not be detected (data not shown).

In the absence of doxycycline but in the presence of untransfected or hCNTF-secreting feeder cells, glial precursor cells became 513^-, $A2B5^{+/-w}$, round flat cells. No long thin $A2B5^+$ cells could be detected.

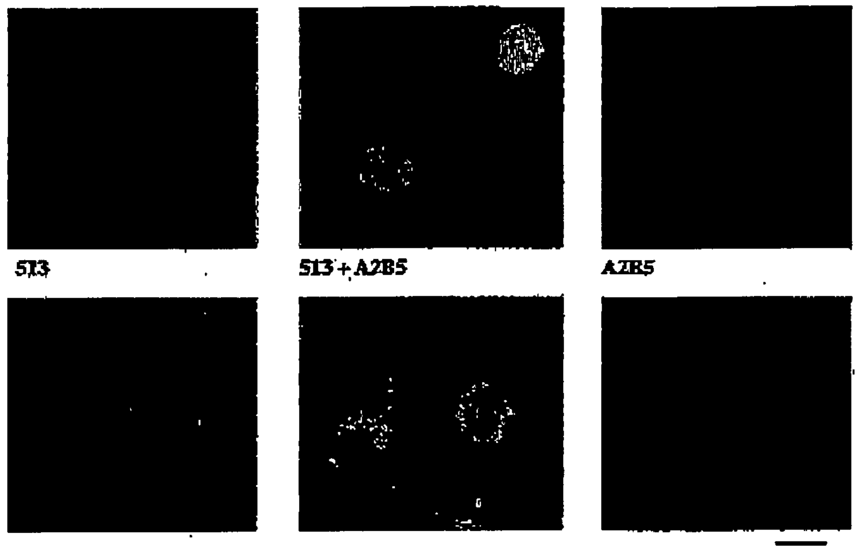
In cultures without doxycycline and in the presence of a hIL6- secreting feeder cell clone, four types of cells could be detected (Figure 7). They are: (1) O2A cells possessing the $A2B5^+$ marker, with long thin spindle morphology, representing a minor population (7%); (2) 513^+ $A2B5^+$ precursor oligodendrocytes, with large nucleus and round cytoplasm (Figure 6, left upper panel, 3 cells), representing a major population (42%); (3) type 2 astrocytes that are $GFAP^+$ $A2B5^+$ with a small nucleus, large irregular cell body, representing 32% of the population; or (4) $GFAP^-$ $A2B5^-$, a large nucleus and cell body, ameba-like cells (19% of the population). Thus, cells co-cultured with hIL6 secreting feeder cells differentiated into precursor oligodendrocytes and type 2 astrocytes.

Four similar types of cells could be detected in cultures

Table 4. The effect of doxycycline and growth factor on the distribution of glial cell subpopulation in co-culturing. Percentage of four glial cell subpopulations upon co-culturing of glial precursor cells with untransfected-, cytokine-secreting tet-off feeder cells. Cells were seeded on coverslips and co-cultured for nine days with or without doxycycline. Gial cells on coverslips were harvested, immunostained and evaluated under a fluorescent microscope

		Percentage (%) of cell types			
Doxy*	Type of tet-off feeder cells	Oligodendrocyte (513^+ and $A2B5^{+/-}$)	O2A precursor (513^- and $A2B5^+$) or intermediate progeny§ (513^- and $A2B5^{-/+w}$)	Type 2 astrocyte ($GFAP^+$ and $A2B5^+$)	Type 1 astrocyte ($GFAP^{+/-}$ and $A2B5^-$) and others (ameba-like)
Without Doxy	No feeder cells	0	100 (dying)†	0	0
	Feeder cells only	0	100 (round cells)†	0	0
	hCNTF-feeder cells	0	>90 (round cells)†	0	ca.10 (others)
	hIL6-feeder cells	42	7	32	19
	hIL3-feeder cells	39‡	4	42	15
With Doxy	No feeder cells	0	100	0	0
	Feeder cells only	0	100 (round cells)	0	0
	hCNTF-feeder cells	0	>90 (round cells)†	0	ca.10 (others)
	hIL6-feeder cells	0	>90 (round cells)	0	ca.10 (others)
	hIL3-feeder cells	0	>90 (round cells	0	ca.10 (others)

*Doxy = Doxycycline.
†No spindle cells found.
‡Some cells have MAG-513^+ inclusion bodies on the surface (Figure 8(a)).
§ −/+w = minus or weak plus.



+LC-Doxy HimrtTA 9d

Figure 6. Confocal microscopic photographs of two fields of cells nine days after co-culturing of glial precursors with hCNTF-secreting feeders. A2B5: mIgM anti-A2B5 plus biotin-G anti-mIgM F(ab)'2 and TR-streptavidin. 513: mIgG anti-513 and FITC-G anti-mIgG. One scale bar = 10 μm

of glial precursor cells grown without doxycycline and in the presence of an hIL3 secreting feeder cell clone (Figure 8(a)). But the progeny is more mature than that derived from hIL6-fed cultures. Namely, there are: (1) O2A cells possessing the A2B5$^+$ marker and with a long thin spindle morphology, representing a minor population (4%); (2) 513$^+$ A2B5$^+$ oligodendrocytes with a large nucleus and elongated cell body and extended processes, representing 39% of the population. On the surface membrane of some such cells, thick round MAG-513$^+$ inclusions were observed. In Figure 7, left upper panel, 2 out of 4 cells have such inclusions. An enlarged inclusion body, sitting on the cell surface, is shown in Figure 8(b); (3) type 2 astrocytes which are GFAP$^+$ A2B5$^+$ and with a small nucleus, little cytoplasm and thin long processes, representing a major population, (42%); or (4) ameba-like GFAP$^{-/+w}$ A2B5$^-$ cells with a large nucleus and cell body, representing 15% of the population.

We think that the differentiation events observed could be attributed to the combination of two events in cultures: (1) the absence of doxycycline, and (2) the presence of feeder cells secreting cytokines such as hIL6 and hIL3. In contrast, feeder cells secreting no transgenic cytokine or hCNTF-secreting feeder cells did not induce such a maturation process. The oligodendrocytes derived from co-culturing with hIL6-secreting feeders seemed to be less mature and differentiated than those co-cultured with hIL3-secreting feeders. They lack long processes and MAG-513$^+$ inclusions on membrane surfaces. The derived type 2 astrocytes also do not exhibit the thin long radial processes. Thus, we postulate that the two elements of appropriate cytokines and removal of doxycycline provide a major differentiation stimulus for the maturation of the glial precursors into progeny.

Discussion

A novel doxycycline inducible glial precursor cell line mimicking primary cells

The data described in the results section suggest that doxycycline provides the stimulus for this cell line to maintain a cycling precursor stage. Withdrawal of doxycycline for a minimal of nine days induced apoptosis in the cells. Withdrawal of doxycycline and the inclusion of untransfected or hCNTF-secreting feeder cells in the same culture did not induce differentiation into progeny. Instead, cells arrested in a round flat, A2B5$^-$, 513$^{-/+w}$ intermediate type. Withdrawal of doxycycline and the inclusion of hIL6-secreting feeder cells in the same culture for nine days induced the differentiation of this precursor cell line into mature type 2 astrocytes and precursor oligodendrocytes. The conditions which produced the most outstanding effect were the withdrawal of doxycycline and the inclusion of hIL3-secreting feeder cells in the culture. The precursor cell line was induced to differentiate into type 2 astrocytes and more mature oligodendrocytes with detectable MAG-513$^+$ inclusion bodies.

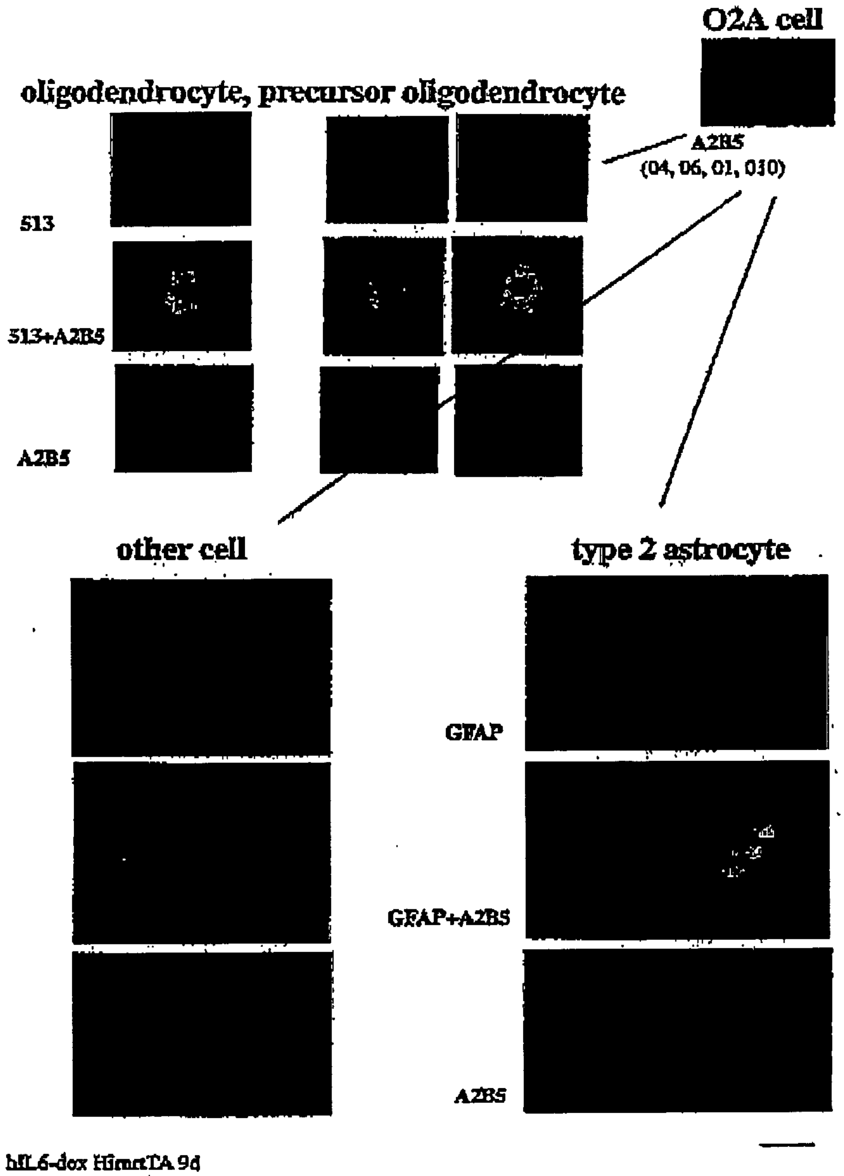


Figure 7. Confocal microscopic photographs of glial precursors co-culture with hIL6-secreting feeders. A2B5: mIgM anti-A2B5 plus biotin-G anti-mIgM F(ab)'2 and TR-streptavidin. 513: mIgG anti-513 and FITC-G anti-mIgG. GFAP: mIgG anti-GFAP and FITC-G anti-mIgG. One scale bar = 10 μm

However, the derived oligodendrocytes still retained a A2B5$^+$ marker. Culturing this glial precursor cell line without doxycycline and with hIL3 secreting feeders for 21 days induced apoptosis in most cells. The few cells still left on the coverslip (ca. 50 cells) seemed to have become mature oligodendrocytes, since they expressed A2B5$^-$, GFAP$^-$, GalC$^+$, although such cells seemed also to be dead (data not shown).

Comparable to primary glial progenitor cells, this cell line expresses A2B5$^+$O4$^{-/+}$ markers, and can differentiate into mature oligodendrocytes and type II astrocytes in culture after inductive and favorable culture conditions are introduced, thus they are O2A-like cells. However, this cell line has several unusual properties. Cells showed a very high background fluorescence when a conventional mAb staining procedure was performed for immunostaining. An extra step to pre-block non-specific binding must be undertaken using a high percentage of

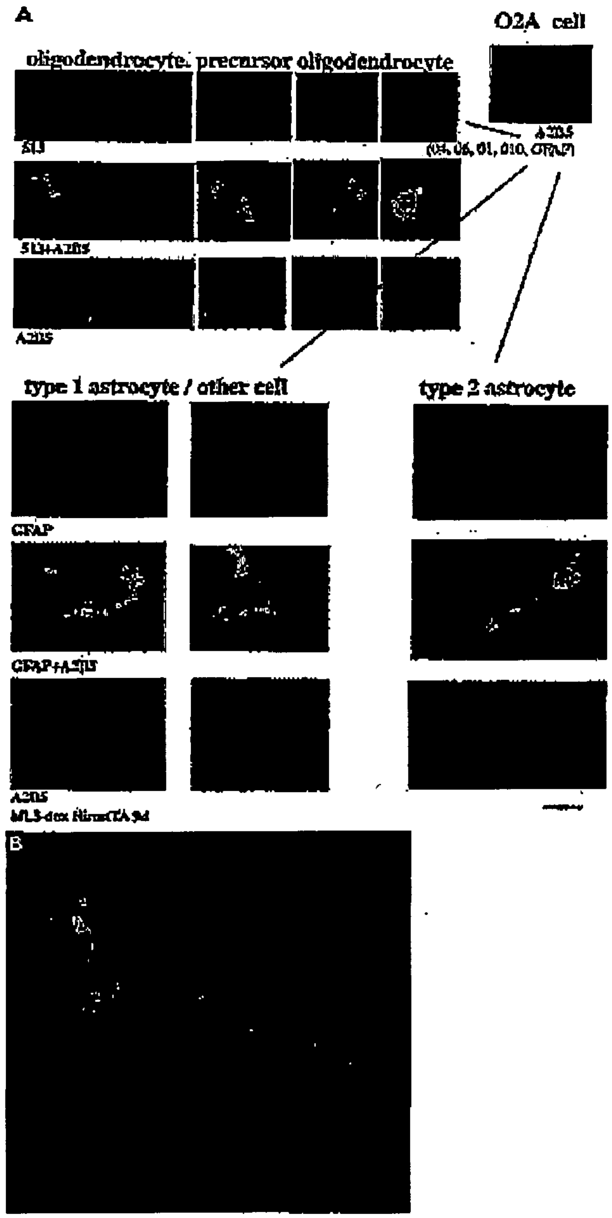


Figure 8. (a) Confocal microscopic photographs of glial precursors co-culture with hIL3-secreting feeders. A2B5: mIgM anti-A2B5 plus biotin-G anti-mIgM F(ab)'2 and TR-streptavidin. 513: mIgG anti-513 and FITC-G anti-mIgG. GFAP: mIgG anti-GFAP and FITC-G anti-mIgG. One scale bar = 10 µm. (b) A confocal microscopic photograph of glial precursor A2B5$^-$ 513$^+$ with inclusion on one cell surface, enlarged from the field of the left upper corner of (a)

protein such as FCS. Cells stained positively with OMP, a marker normally expressed in the olfactory neural lineage, and they expressed cytoplasmic NF68KDa$^+$ and cytoplasmic instead of nuclear Oct-6$^+$. They were also O1$^+$ and O10$^+$; both are markers which are thought to be expressed in cells further downstream of gliogenesis during the development of primary glial cells.

In this study, the electrophysiological measurment of these cells using the patch clamp technique has shown a membrane potential and current patterns similar to that of

cultured primary glia cells. However, under the current culture condition and in the buffer conditions used in this measurement, the cells investigated electrophysiologically were not found to be excitable. Further measurement will be performed using cells freshly isolated from animals implanted with this cell line for a known period (in progress).

Despite the fact that these cells demonstrated unusual membrane potential, the results of electrophysiology are consistent with other data and suggest that this cell line is possibly of O2A cells. Based on the electrophysiological and morphological criteria of glia, the known glial markers expressed, and its differentiation potential *in vitro*, this doxycycline inducible glial precursor cell line seems to correspond to the 'O2A cell' in the lineage development of gliogenesis, as summarized in a cartoon (Figure 5).

Proliferation versus differentiation, cis- versus trans-differentiation

It is unknown how the cells decide whether to stay in the stem cell compartment or begin to differentiate. It is also not known whether the proliferation and differentiation events are coupled or uncoupled events, i.e., whether cells stop proliferating and if they do stop proliferating, what then happens to such stem cells? Do they remain as stem cells, or will they die, or will they begin to differentiate? How does the differentiation event occur, stochastically or inductively [21]? Such questions have long been asked. Using novel approaches to yield results such as those described in this study allows us to address the problems at the cellular level, and eventually at the molecular level.

With this doxycycline-dependent glial precursor cell line, whether cells proliferate or arrest in the G_1 phase can be controlled at will. Moreover, we have demonstrated in this study that we can regulate the status of the cell cycle. As shown in the results section, when the cells cease to proliferate after withdrawing doxycycline, the cells die. The differentiation process does not occur automatically. It is induced by external cytokines constantly supplied by co-cultured feeder cells. The degree of differentiation and the types of matured progeny depend very much on the various kinds of cytokines present. Preliminary attempts using an approach similar to Kondo and Raff [22], may result in cell types of neuron lineages (data not shown). With this approcah, we should be able to further isolate intermediate cells corresponding to those of glio-differentiation (Figure 5). It means that events deciding whether cells are at the intermediate stage of differentiation, or whether they have reached the mature progeny of cis-, trans- or retro-direction, could be determined, and expanded as clones (work in progress). Samples can be collected kinetically to determine cells and genes of interest expressed in particular lineages. Thus, this is a very powerful system for analyzing the molecular events underlying the direction of differentiation.

Future applications in the field of gene delivery

Cell lines capable of differentiating into neurons, astrocytes, and oligodendrocytes have been established from human, rat, and mouse [23–25]. Among them, temperature sensitive (ts)-SV40Tag immortalized glial precursor cell lines from ED14 mouse brain were established [7–9] and transplanted to treat rat EtBr demyelinating lesions. A growth factor-dependent rat oligodendrocyte precursor (CG4) was shown to remyelin *md* rat [26,27]. Two of the immortalized cell lines are being applied in current clinical trials for treating ischemic stroke. A pluripotent tumor cell line isolated from testicular teratocarcinomas of a metastasis patient has been shown to stop proliferating and to differentiate into neuronal cells using retinoic acid, to improve recovery in an animal model, and is presently being transplanted into patients with lacunar stroke [28–30]. A neuroepithelial stem cell immortalized with ts-SV40 T antigen has been shown to have positive effects on the sensory neglect and motor asymmetries in a rodent model [31], and is currently undergoing clinical trial in the UK.

The cell line established and described in this communication is different from the published cell lines, in that it is inducible to proliferate under the control of external signal-doxycycline *in vitro* as well as *in vivo*. When cells are grown in the presence of doxycycline, either in culture medium (in Results section) or implanted into mice and fed with doxycycline containing drinking water, the cells will proliferate. Without doxycycline in culture (in Results section), or in the drinking water, cells stop proliferation (work in progress). Further characterization of the biological properties and function of this cell line *in vitro* and *in vivo* is in progress. We also predict that this cell line may provide a tool for identifying and characterizing novel genes involved in cell division and the various steps of glio-genesis. It may also have an application in the delivery of cell-based neurotrophic factors to the CNS conditionally, for treating neurodegenerative and other diseases, when factors are used as the transgene (in progress).

Acknowledgements

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Characterization of a mouse tet-on glia precursor cell line in vitro and in vivo using the electrophysiological measurement

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Abstract

We report here a partial characterization of a "tet-on" glia O2A precursor cell line established from the reverse tetracycline-transactivator (rtTA)-SV40 T antigen (Tag) double transgenic mice. In culture, withdrawal of doxycycline prevents proliferation and the cell line undergoes apoptosis. Importantly, differentiation into type-2-astrocytes and oligodendrocytes can be induced when the cell line is cultured, in the absence of doxycycline, and with epithelial stem cell lines secreting hIL3 or hIL6. In contrast, no maturation into progeny was observed when a hCNTF-secreting cell line was used as the co-culture partner under the same condition. In order to address the question of whether the morphological distinct cells—spindle and stellar shaped cells are of a similar or different cell types, we have performed cell size analysis of these cells by FACS and electro-physiology measurement by the patch clamping technique. They are of a similar cell size, but posses distinct electrophysiological properties—spindle cells are less mature than the stellar cells. These tet-on glia O2A precursor cells were implanted to sites of transected sciatic nerve of adult mice and kept in the precursor stage by feeding mice with doxycycline containing drinking water. The toe movement of injured foot was measured every 3 weeks and the electrophysiological property of motor neuron was determined three months after the operation. Preliminary data have shown that these tet-on glia precursor cells are not toxic to the implanted hosts and can enhance the recovery of damaged motor nerves.

Keywords: tet-on O2A glia precursors; Electro-physiology

1. Introduction

Having the property of self-renewal, stem cells offer the promise of lifelong repair of damaged tissue. However, somatic tissue-committed primary stem cells are rare and difficult to expand in vitro. Genetically modified stem-like cells with the ability to expand conditionally provide a valuable tool with which to study stem cell biology. In addition, the cells may be appropriate candidates for establishing an animal model for designing therapeutic strategy and clinical applications.

Neuronal-glial pluro-potent stem cells of rodent and human embryonic origins can grow in the sphere form at the presence of growth factors. However, human adult neuronal stem cells are difficult to expand under similar culture condition. Moreover, after the pluro-potent stem cells committed to neuronal lineage, expressing adrenergic or dopaminergic lineage markers, either at the precursor stage or at downstream mature neuron stage, are unlikely to be expanded and kept in

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the proliferative and/or non-apoptotic condition. It is postulated that glia cells may play an important role to support the expansion, survival and function of such (precursor) neurons. It would be highly desirable to establish an universal supporting glia cell line for such a purpose. In this communication, we further characterize a doxycycline inducible (tet-on) glia precursor cell line in vitro and in vivo, which has the capacity to differentiate into mature astrocytes and (precursor) oligodendrocytes. In preliminary attempts, this cell line has shown promising capacity to enhance the repair-regeneration of damaged adult peripheral neurons when implanted in vivo.

2. Materials and methods

Double transgenic mice of NMRI strain, possessing SV40 Tag and under the control of the reverse tetracycline-transactivator (rtTA), were used to establish cell lines. A brain cell line was partially characterized in vitro by morphology, immuno-staining and confocal microscopy, cell size determination, and electro-physiological methods using the patch clamp technique.

2.1. Immuno-staining of cells and confocal microscopic image analysis

To measure the epitopes expressed in the glia lineage, we performed immuno-fluorescent labeling with antibodies and evaluated using confocal microscopy [1]. Cultured cells on coverslips were fixed with 4% paraformaldehyde in PBS at room temperature (RT) for 30 min. They were washed twice with PBS (in between steps), nonspecific binding sites were blocked with 2% FCS (Fetal Calf Serum) in PBS at RT for 1 h, then stained with the following antibodies to identify several epitopes of glial lineage: 513: mIgG anti-513 (gift of J. Trotter, 1:20 dilution) and FITC-goat (G) anti-mIgG (Jackson, West Grove, 1:100 dilution); A2B5: mIgM anti-A2B5 (Boehringer, Mannheim, 5 µg/ml), biotin-G anti-mIgM F(ab)'2 (SBA, Birmingham, 1:100 dilution) and Texas Red (TR)-streptavidin (Amersham, Amersham, 1:100 dilution; GFAP: mIgG anti-GFAP (glial fibrillary acidic proteins, Boehringer, Mannheim) and FITC-G anti-mIgG. Afterwards, the cover slips were embedded in Moviol containing 100 µg/ml DABCO (Vectashield, Bector, Burlingame).

Immuno-fluorescence was evaluated using confocal scanning laser microscopy. A Leica DM IRBE microscope equipped with a 63×Plan Apochromate objective was used in this study. Analysis of the antigens, stained by using the TR- and FITC-labeled secondary antibodies was performed, by using the TRITC- and FITC-fittings at a pinhole size of 0.45. Relative amplification of the FITC and TRITC-emissions was 1:0.97. Laser excitation energy and amplification of FITC- and TRITC-emissions were left constant within each experiment to allow the comparison of different samples.

2.2. FACS measurement to estimate cell size

Cell size analysis was performed on living cells harvested after trypsinization. Flow cytometry analysis was performed on FACScan (Becton Dickinson).

A total of 20 000 cells in each sample were acquired. FSC (Forward Scatter Count) histogram plots are shown for each cell sample, overlaying the parental cell line. For each cell clone, the percentage of cells in region M1 (180–440 FSC units), and in region M2 (440 and 1000 FSC units) was estimated.

2.3. Trangenic glia precursor cells with cytokines

Construction of plasmids coding for hIL3 (human Interleukine 3), hIL6, hCNTF (human Ciliary NeuroTrophic Factor), the transgenic insertion of plasmid, selection of positive stable insertion clones, and the measurement of secreted cytokines in culture supernatants have been described [2].

2.4. Patch clamp technique to measure membrane currents

As previously described [1,3], cover slips with adhered cells were placed in a chamber mounted on the stage of a Zeiss microscope (Axioplan, Zeiss, Oberkochen, Germany) and fixed in the chamber using a U-shaped platinum-wire for electrophysiological recordings. The chamber was continuously perfused with HEPES buffered bath solution and substances were added by changing the perfusate. Membrane currents were measured with the patch-clamp technique in the whole-cell recording configuration [4]. Current signals were amplified (EPC-9 amplifier, HEKA, Lambrecht, Germany), filtered at 3 kHz and sampled at 5 kHz by an interface (HEKA) connected to a PC system which also served as a stimulus generator. All patch clamp data analysis was performed using the WinTida software package (HEKA). The resistance of the patch pipettes was 5–6 M. The bathing solution contained (in mM) NaCl, 150; KCl, 5.4; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 5; and glucose, 10. The pH was adjusted with NaOH to 7.4. The pipette solution contained (in mM) KCl, 130; $CaCl_2$, 0.5; EGTA, 5; $MgCl_2$; HEPES, 10, Ca^{2+} activity was calculated to be approximately 11 nm. The pH was adjusted with KOH to 7.2.

2.5. Sciatic nerve injury and implantation of cells

Twelve–14-week-old NMRI mice are used in this study. The mice were anaesthetized with Fentanyl

citrate fluanisone and left side sciatic nerve was transected using a scissors [5]. O2A precursors were seeded onto a denuded brain (DEB) tissue. The condition of cell seeding is ca. 1×10^6 cells/cm^2 of DEB in a petri dish, filled the cell-DEB tissue with a minimal amount of RPMI 1640 medium with 10% FCS and at the presence of 1 μg/ml doxycycline. The cell-DEB tissue was incubated overnight in a 37 °C incubator and transplanted into the site of sciatic nerve injury before the closure of the wound.

2.5.1. Feeding with doxycycline containing drinking water

In order to keep the implanted cells in the proliferating precursor cell stage, mice were given drinking water containing doxycycline 20 μg/ml dissolved in 5% glucose, following the protocol described in [6]. It started 2 days after the implantation and continued through the entire experimental period.

2.5.2. Score of toe movement of the injured foot

Every 3 weeks, the experimental mice were lifted in the air, then let run freely. The degree of toe-bending and movement was recorded. The range of movement is scored arbitrarily as 1, 25, 50, 75, and 100° comparing to the non-transected side of the toes. In details, 1° means that toes were straight, with no movement. 25° means that toes could bend very weakly, with minute movement. 50° means that toes could bend gently, with slight movement. 75° means that toes could bend but with some difficulty, but easy movement, and 100° is that toes could bend freely, with full movement. The means for each group of mice were calculated and differences were tested with Student's *t*-test.

2.5.3. In vivo electro-physiology

Electrophysiological measurement was performed 3 months after the operation, following the procedure described earlier [7]. The mice were lightly anaesthetized with Fentanyl citrate fluanisone to suppress voluntary activity and pain reactions to needle insertion. A concentric needle electrode (0.5 mm diameter) with a lead-off area of 0.03 mm^2 was advanced through the lateral abdominal wall to the right diaphragm, and the electromyogram was recorded. Care was taken not to record from inter-costal muscles and not to penetrate the diaphragm. For each mouse, 5–15 inspiratory bursts recorded from at least two sites were analyzed; duration, maximal amplitude, and number of negative spikes of at least 30 μV amplitude were determined. The means for each group of mouse were calculated and differences were tested with Student's *t*-test.

Compound action potentials of the calf muscles were elicited by supra-maximally stimulating the sciatic nerve at the sciatic notch through non-insulated platinum needle electrodes and were recorded between two electrodes placed over the belly of the anterior tibial muscle and at the ankle. Shape and amplitude of the potentials did not change when the recording electrode was moved to the gastro-cnemius muscle; this indicated that the recorded potential was the combined extensor and flexor muscle potential. Peak-to-peak amplitudes and the motor latencies were measured. All stimulation and recordings were done with a key-point electromyography (Dantec, Copenhagen, Denmark).

3. Results

We report the partial characterization of a cell line with two distinct cell morphologies (Fig. 1) derived from the brain tissue of rtTA-SV40 Tag transgenic mice. Analysis of the cell fate and antigen markers have indicated that this cell line mimics some aspects of primary O2A glia precursors, as drawn in a cartoon (Fig. 2 [8]). Cell cycle progression of this cell line is doxycycline-dependent [1].

3.1. Progenies matured upon co-culturing of O2A precursors with cytokine-secreting feeder cells

In order to address the question of whether these precursor cells can differentiate into mature progeny in culture such as oligodendrocyte, type 2 astrocytes, or type 1 astrocytes (Fig. 3, left panel), we perform ten different cell co-culture conditions. Morphology of mature cell progenies and the percentage of mature cells in each set of culture stimulation are summarized in the right panel of Fig. 3. In the absence of doxycycline, O2A precursor glia cells become apoptotic, and no mature cell types can be found (culture condition 1). Co-culturing these cells with epithelial stem cells alone and with/without doxycycline in culture medium, no mature cell types are found neither (culture condition 2, and 6). When hCNTF-secreting epithelial stem cells served as feeder cells, they do not induce the O2A precursors to mature into progeny cell types (culture con-

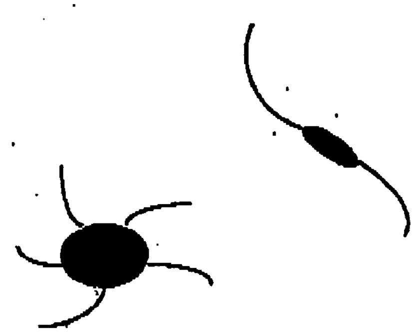


Fig. 1. Cell division pattern: the picture was drawn based on the morphology of the two cell types observed under the microscope.

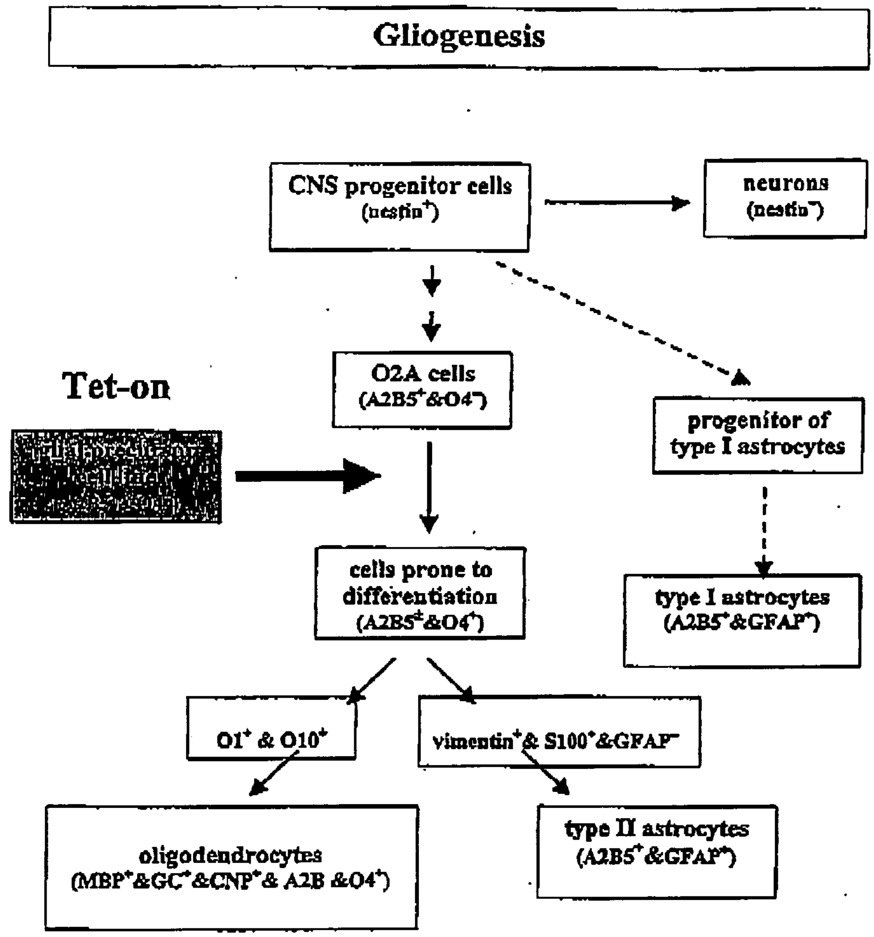


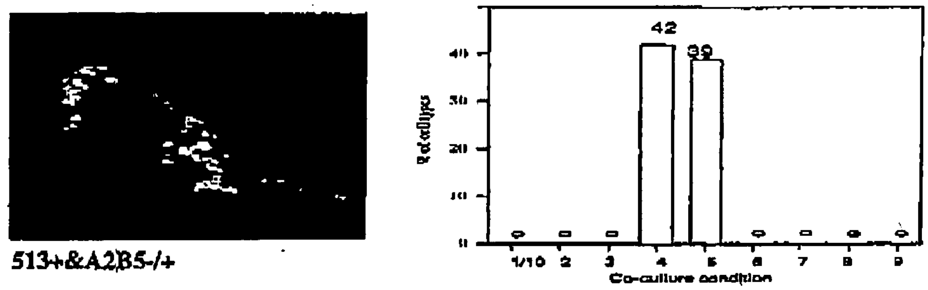
Fig. 2. Gliogenesis: a doxycycline-inducible cell line, possibly of O2A cells or the immediate progeny. The lineage development of glial cells drawn in the figure is based on published literature.

dition 3, and 7). Differentiation into (precursor) oligodendrocytes and mature astrocytes can be induced upon withdrawal of doxycycline and addition of epithelial stem cells secreting cytokine such as hIL3 (culture condition 5), or hIL6 (culture condition 4) to the culture. The percentage of mature cell types varies from 32 to 42% for oligodendrocytes and type 2 astrocytes, and 15 to 19% for cells with morphological characteristic of type I astrocytes.

3.2. Cell size measurement by FACS analysis

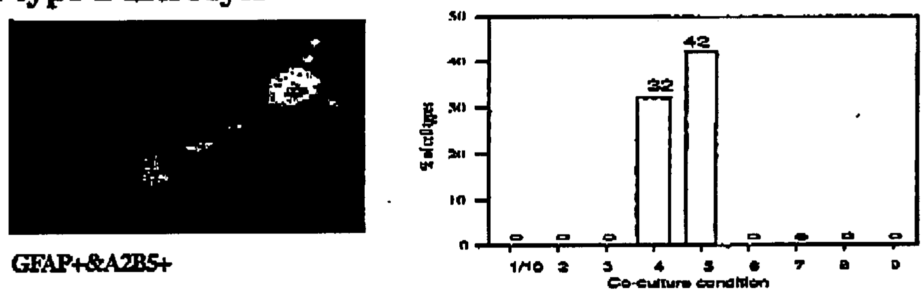
In culture, two types of cells could be observed under the microscope: (1) flat cells with a round cell body (drawing in Fig. 1, left) and (2) long cells with an elongated thin cell body (drawing in Fig. 1, right). In order to determine whether these two types of cells are of the same or different size, we perform the FACS of the living single cells after the cells detached from petri dishes into suspension by trypsinization. The cell size of four different transgenic cell clones, which contain GFP (Green Fluorescent Protein), hCNTF, hIL3, and hIL6, was compared to the parent cell line (Fig. 4). The data show that hCNTF- and hIL3-O2A cell clones (Panels B and C) display more small sized events than the other two transgenic cell clones, GFP- and hIL6- O2A (Panels A and D), which are possibly of debris and/or apoptotic bodies. With respect to size, the data also show that two subsets can be distinguished in all cell clones, one in region M1 (180–440 FSC units), and one in region M2 (440–1000 FSC units); the proportion of cells in each size region differs depending on the cell clones. Cell clones GFP-O2A and hIL6-O2A (Panels A and D) are very similar to the parental cell line O2A. Cell clones hCNTF O2A and hIL3-O2A (Panels B and C) exhibit relatively more cells of the lar-

A. (precursor) oligodendrocyte



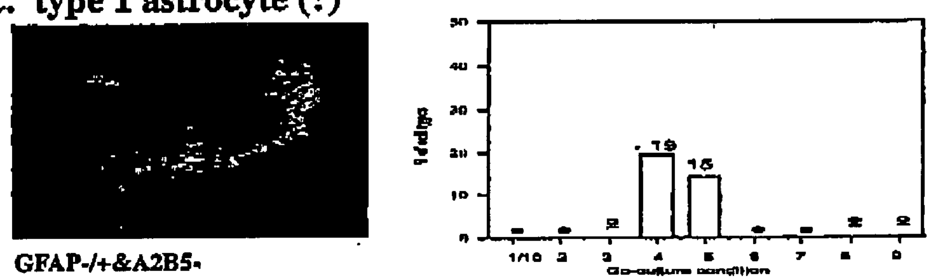
513+&A2B5-/+

B. type 2 astrocyte



GFAP+&A2B5+

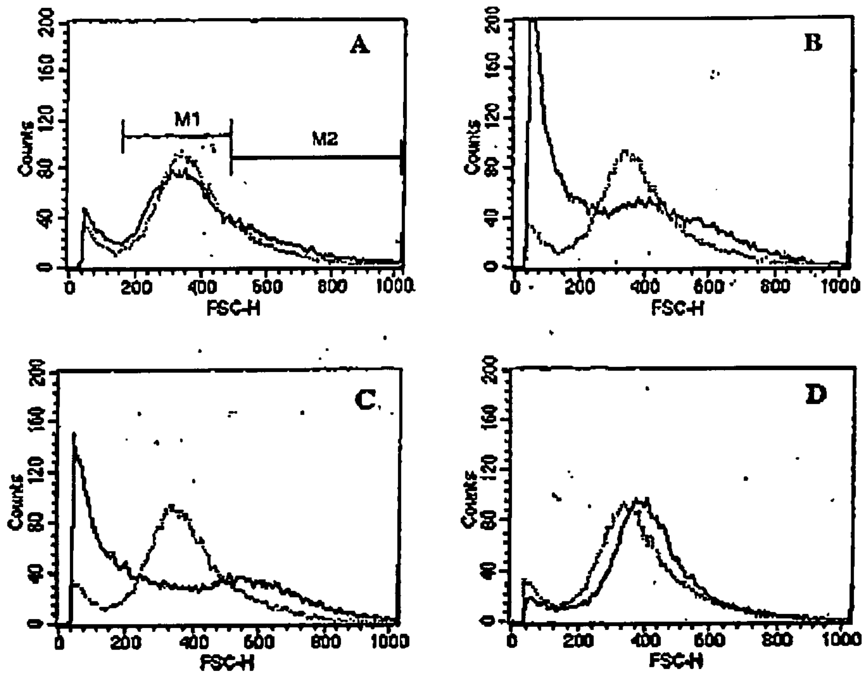
C. type 1 astrocyte (?)



GFAP-/+&A2B5-

1. no feeder cells - Doxy
2. feeder cells only - Doxy
3. hCNTF-feeder cells - Doxy
4. hIL6-feeder cells - Doxy
5. hIL3-feeder cells - Doxy
6. feeder cells only + Doxy
7. hCNTF-feeder cells + Doxy
8. hIL6-feeder cells + Doxy
9. hIL3-feeder cells + Doxy
10. no feeder cells + Doxy

Fig. 3. Left panel. Confocal microscopic photographs of three mature cell types derived from glia precursors after co-culture with hIL3 secreting feeders. A2B5: mIgM anti-A2B5 plus biotin-G anti-mIgM F(ab')2 and TR-streptavidin. 513: mIgG anti-513 and FITC-G anti-mIgG. GFAP: mIgG anti-GFAP and FITC-G anti-mIgG. One scale bar = 10 μm. 3(A) (precursor) oligodendrocyte: a confocal microscopic photograph of glia precursor A2B5$^{\pm}$ and 513^{-} with myelin-like inclusion on cell surface. 3(B) type 2 astrocyte: a photograph of a star shape cell possessing GFAP^{+} and A2B5^{+} markers, called type 2 astrocyte. 3(C) type 1 astrocyte: a large irregular shape cell possessing GFAP$^{\pm}$ and A2B5-markers, possibly of type 1 astrocyte. Right panel. Percentage of mature glia cell types found in culture containing various kind of (cytokine-secreting) feeder cells. The 10 different cell co-culture conditions are listed at lower right corner.



Panel	Cell clone (x-O2A) x=	M1 (%)	M2 (%)
A	GFP	73	27
B	hCNTF	66	34
C	hIL3	55	45
D	hIL6	77	23
A-D (dot line)	O2A parental	82	18

Fig. 4. Cell size distribution using FACS technique. Panel A: GFP-O2A, Panel B: hCNTF-O2A, Panel C: hIL3-O2A, and Panel D: hIL6-O2A, dash dots in all panels: O2A, M = mean value.

ger size. In all events, the cell population of different transgenic insertion has the same size range. Thus the total cell-surface area and possibly the cell volume are similar despite the morphological distinct figure of these two kinds of cells.

3.3. Electrophysiology measurement in vitro using a patch clamping technique

In order to differentiate these two cell types, they were further analyzed using the patch clamp technique. Cells were grown on coverslips instead of plastic petri dishes. The thin long cells were the majority under culture conditions, where proliferation had been stopped 2 days before the electrophysiological investigation by withdrawing doxycycline from the culture medium. For measurement, cells were growing on un-coated glass coverslips. Unlike primary glial cells freshly isolated from mice, cells of this line did not grow well on poly-L-lysine (PLL)-coated coverslips. The non-proliferating cells adhered slightly better on PLL-coated coverslips, and on such coverslips, the long thin cells seemed to occur more frequently.

Besides the morphological differences, electrophysiological differences between the different cell types could be observed among 40 cells examined, but the patterns did not correlate to 100%. The cells displaying a long thin morphology did exhibit activating currents in some cases (Fig. 5, Panel C). The flat cells exhibited a passive conductance (Panel D) sometimes displaying tail currents. In total, five cells displayed significant tail currents. The cell shown in Panel A was patched with a pipette solution containing lucifer yellow. In Panel B, it shows the coupling of the two cells. In addition, the

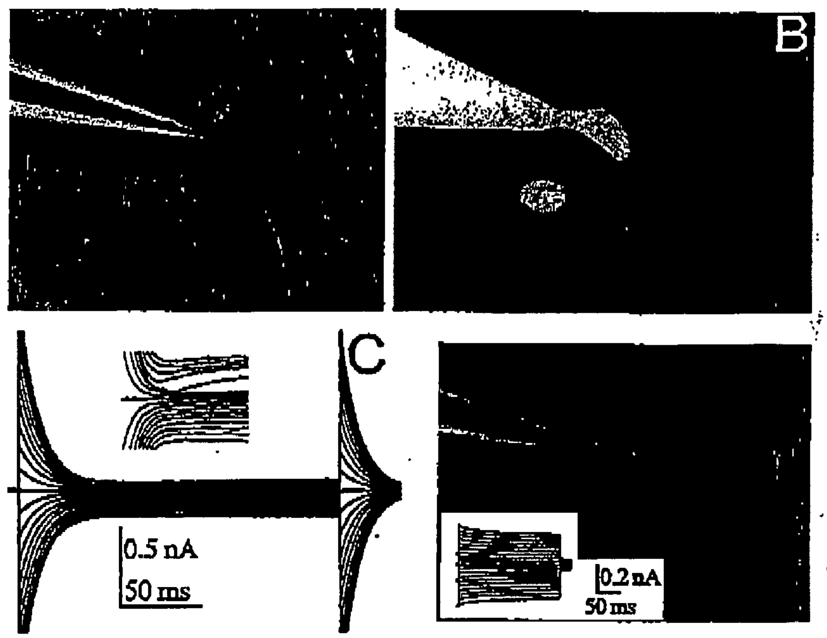


Fig. 5. Electrophysiological properties of the cell line in vitro: Panel A. A thin long cell and a round flat cell are shown being patched for electrophysiological property. Only the thin long cell was patched with a pipette containing lucifer yellow in the solution. Panel B. The coupling of a thin long cell and a neighboring round flat cell (of Panel A) is shown. Lucifer yellow was used as an indicator to inject the thin long cell. Panel C. The current pattern of the thin long cell patches in Panel A (and Panel B) is shown. The pattern has outwardly rectifying properties. In the pipette, Cs^{+2} and TEA were included in the solution in order to block passive potassium-conductance of the cell. Panel D. A round flat cell is shown being patched. Insert in Panel D: 10×enlargement of the picture shows that this cell expressed a delayed rectifier current. It shows that depolarizing and hyper-polarizing voltage pumps resulted in symmetrical currents with no apparent time-dependence.

pipette solution contained Cesium^{+2} (Cs^{+2}) and tetra-ethyl-ammonium (TEA) to block passive conductance. The data imply that gap junction channels may be responsible for the coupling of these two cells, allowing the intercellular spread of lucifer yellow. The molecular nature of gap junction channel will be analyzed in the near future.

This long thin cell expresses a delayed rectifier current (inset in Panel C). All cells investigated electro-physiologically were not excitable. The expression of mainly passive currents, delayed and inward rectifier currents in some cases and the existence of tail current points to glia properties of these cells. In addition some of the flat cells do show an oligodendrocyte-like morphology. The electro-physiological data are consistent with other cellular analytical data ([1] and above), and suggest the cell line being derived from O2A glial precursor cells (drawing in Fig. 2).

3.4. Role of tet-on glia precursor cells in vivo

In order to test whether this cell line possesses the capacity to interact with neuron, by influencing the degree of repair of damaged motor neuron, we decided to use the injured sciatic nerves of adult mice as the study model. Sciatic nerve of adult mice were transected mechanically using a scissors, and O2A glia precursors seeded onto DEB (Denude brain) tissue were implanted in situ before the closure of wounded sites. Mice were fed with doxycycline in drinking water [6] in attempting to keep the tet-on glia precursor cells in the proliferating and non-differentiated stage [1]. The moving behavior

Table 1
The effect of implanted O2A precursor cells on the degree of toe movement after sciatic nerve injury

Time point[b]	Arbitrary degree of toe movement[a]									
	Cells implanted					No. cells implanted (control)				
	Mouse no.									
	1	2	3	4	Mean	5	6	7	8	Mean
1	1	50	50	100	52.7	1	1	1	1	1
2	25	75	75	75	62.5	25	1	50	75	37.5
3	75	50	75	100	75.0	25	25	75	50	43.7

[a] 1°: toes were straight, with no movement; 25°: toes could bend very weakly, with minute movement; 50°: toes could bend gently, with slight movement; 75°: toes could bend but with some difficulty, with easy movement; 100°: toes could bend freely, with full movement.
[b] A 3-week period was separated for each measurement.

of mice was monitored and recorded periodically for up to 3 months. Besides the handicap leg, no obvious suffering or abnormal behavior was observed in these two groups. As shown in Table 1, arbitrary units were designated to quantify the degree of toe bending and toe movement in injured and control mice. It was scored between the group of mice receiving glia precursor cell implant and those receiving no cell implant. After the operation, the toes of the group of mice receiving cell implant seemed to move better than those receiving no cell implant—a 52.7-fold higher score at time point 1. The transected sciatic nerves in both mouse groups recovered with the time. But the degree of toe movement of the group of mice receiving cell implant was still higher than those receiving no cell implant, i.e., 1.67-fold at time point 2, and 1.71-fold at time point 3. Student t-test has revealed values of $P = 0.017$, 0.07 and 0.014, indicating that there is a difference between these two groups.

Electro-physiology of motor neuron was measure 3 months after the operation. As shown in Fig. 6, the percentage of recovery of two groups of mice, with and without implantation of glia precursors were compared. The mean values ±Standard Deviation (S.D.) of both group were calculated to be 44.50 ± 42.69 for the group of mice implanted with glia cells and 21.75 ± 29.32 for the control group of mice with no cell implant. There is a 2-fold difference of the group with cell implants and with no cell implant, in terms of the recovery of the electro-physiology activity at this particular time point. These data match well with the behavior data of toe bending and movement (Table 1, time point 3). The t-test was performed and revealed to be $P = 0.413$ with two sample t-test; or $P = 0.386$ with Wilcoxon rank sum test.

These preliminary results imply that cell implant helped an earlier and freer toe bending and movement during the earlier stage of recovery from sciatic nerve injury. It appeared also an enhancing effect to improve the movement. However, sciatic nerve injury recovers automatically, so that at the later stage of recovery, there is no drastic difference between these two groups.

4. Discussion and conclusions

The differentiation of this O2A glia precursor line does not occur automatically in culture. Withdrawal of doxycycline from the culture medium removes the proliferation and induces a fatal outcome. Two events, withdrawing doxycycline from culture and additional external help from the cell-based delivery of cytokines, are required for this cell line to differentiate into progenies.

In this communication, two aspects of questions addressed are—how do the tet-on O2A precursor cells behave in culture and in animal?

4.1. In culture

In this study, the method to measure membrane currents was the patch clamping technique. It has revealed

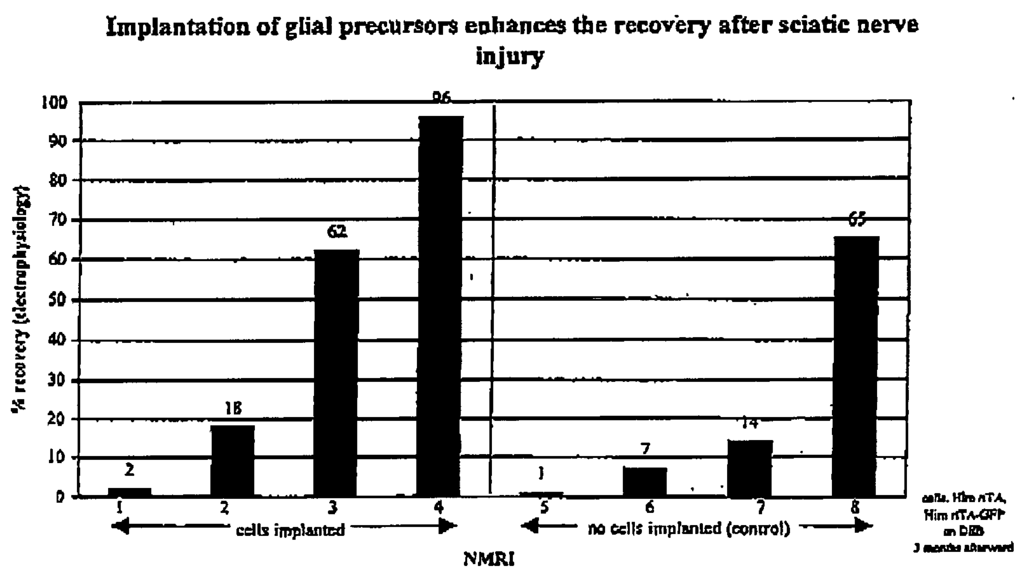


Fig. 6. Percentage recovery of injured sciatic nerve with/without the implantation of tet-on glial precursor cells as determined by electrophysiological measurement.

fine details of these two types of O2A glia precursors. The spindle shape cells appear to be the immediate precursor of the stellar shape cells, when comparing the results of this cell line with that of primary cells freshly isolated from animals. The cell-cell coupling is visible using a dye—lucifer yellow. The molecular nature of the channel of membrane communication remains to be elucidated.

In culture, the precursor cells can mature into progeny—oligodendrocytes which contain 513^{+} myeline-like inclusion body and type 2 astrocytes. This differentiation event occurs if the cells stop proliferation by removing doxycycline and culture together with hIL3- or hIL6-secreting feeder cells. The mature cells are characterized using immuno-fluorescent staining and confocal microscopy. The question of whether the derived progeny possesses a similar electrophysiological property as the counter part of primary astrocytes and oligodendrocytes remains to be answered.

4.2. In animal

It has been documented that one type of mature glia cells, i.e., astrocytes, secrete a rich panel of soluble substances such as CNTF, GDNT, cytokine, neuro-transmitter, etc. [9,10]. These cells also express a variety of receptors and ion channels [11–13]. It is unknown whether the O2A precursor glia possesses a similar property and secretes substances of similar kinds. The amount of soluble molecules secreted from this cell line is, however, not in a sufficient quantity to be detected in culture supernatant using conventional ELISA assays (data not shown). RT-PCR using primers coding for a wide range of genes for soluble molecules is recommended in the future experiments to determine the nature of such secreted molecules from this cell line. It is also well documented that glia-neuronal interaction plays a critical role in the repair and regeneration of damaged neurons.

To study glia-nerve cell interaction, we have chosen to investigate whether the tet-on O2A precursors have an effect in peripheral nerve repair using adult syngenic mice as the animal model. The assumption is that either the glia precursor itself or the array of secreted soluble substances can modulate the repair of transected sciatic nerve. To use syngenic mice as hosts for implantation, we avoid the complication of immune rejection of implanted cells. We show that there is a differance of toe movement between the group of injury mice receiving cell implantation and the control group of injury mice, which did not receive the cell implants. The glia cell implant seems to improve the degree of toe bending and movement of the transected mice as judged by using the behavior assay. However, the transected peripheral nerve can regenerate with the time and there is an extremely high degree of variation of recovery among individual mice using the electrophysiological assay. Statistically it becomes difficult to show a drastic improvement of toe bending and movement between these two groups and/or higher electro-physiology value of the leg muscle in injured mice due to cell implants.

However, from the strategic point of view, in attempting to use cells for implantation in vivo for therapeutic purposes, it becomes important to address questions such as, whether the candidate cells are toxic to the animal? Whether the cell implant inhibits the recovery of transected sciatic nerves? To both questions, this tet-on glia precursor cell line is neither toxic to the experimental hosts, nor inhibitory to the recovery of damaged peripheral nerves of the host animals. These are very valuable observations, since another epithelial cell line secreting a transgenic growth factor was shown to hinder the recovery of injured facial nerve in another set of experiments (U. Chen, H. Schmalbruch and M. Sendtner, unpublished data).

How do these O2A glia precursor cells behave in vivo? When doxycycline is included in the drinking water for mice, what is the fate of such cells? Do these tet-on glia precursors remain in the precursor stage, or do they differentiate into mature oligodendrocytes and astrocytes in the microenvironment of the damaged sciatic nerve? Is the pre-seeding of cells to DEB for implantation really required? Which cell types of the glia lineage and/or their derived substance contribute to the enhanced recovery effect? What will happen if doxycycline will not be included in the drinking water? Such questions need to be addressed and investigated further. Mechanical transect of sciatic nerve requires intensive and excellent surgical operation. Also, a large number of mice and various transgenic cell clones are recommended for each set of experiment to address each above question and to obtain results with statistic significancy. Because of such technical consideration, we are undertaking the task to breed mutated animal, for example, *pmn* (progressive motor neuronopathy) mice [14] as the alternative models.

In summary, we have shown in this communication that the tet-on O2A glia precursor cells consist of a spindle-shape precursor cell type and a stellar-shape immediate progeny cell type. In vivo, these cells are not toxic to the implanted animal, and can somehow enhance the earlier recovery of the toe bending and movement caused by peripheral nerve injury in adult mice.

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